AD_____

Award Number: DAMD17-03-1-0155

TITLE: Proteomic Analysis of Cisplatin-Resistant Ovarian Concers

PRINCIPAL INVESTIGATOR: John Turchi, Ph.D.

CONTRACTING ORGANIZATION: Wright State University
Dayton, OH 45435

REPORT DATE: March 2006

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-03-2006	Final	1 MAR 2003 - 28 FEB 2006

4. TITLE AND SUBTITLE
Proteomic Analysis of Cisplatin-Resistant Ovarian Concers

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-03-1-0155

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
John Turchi, Ph.D.

E-mail: jturchi@iupui.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Wright State University
Dayton, OH 45435

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

One of the major clinical challenges in the treatment of ovarian cancer is that the cancer cells are, or become, resistant to the drugs used to treat the disease. When the cell no longer responds to the drugs, the cancer continues to grow unabated. Some cellular factors that contribute to making a cell resistant to chemotherapy drugs have been identified, though many still remain to be discovered. These cellular factors or proteins involved in drug resistance can be measured using sensitive analytical techniques. A major goal of the research proposed in this study is to analyze these proteins from ovarian cancer cell lines that are known to be either sensitive or resistant to the chemotherapeutic drug cisplatin, a first line treatment for ovarian cancer. We will determine if there is a specific protein "fingerprint" that is indicative of either sensitivity or resistance to cisplatin. Once the useful factors that influence drug resistance are identified in cell lines and verified using tumor biopsies, we anticipate that this information could then be used to help predict whether a specific tumor will respond to a specific treatment. To date, the sensitivity of a specific ovarian carcinoma to a specific treatment can only be assessed by administration of the treatment and then observing the outcome. Knowing the factors that contribute to a cancer being sensitive or resistant and having the methods to determine if these factors are present or absent in a given tumor are the goals of this proposal. This information could then be used in the clinical assessment to determine the best course of treatment for a specific cancer.

15. SUBJECT TERMS
No subject terms provided.

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
					USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
U	U	U	UU	9	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

Introduction

A major impediment to successful treatment of ovarian cancer is clinical resistance to chemotherapeutic agents (1). In addition, despite recent advances (2;3), difficulties in detection of ovarian cancer result in most women presenting with advanced disease (4). Diagnosis of resistant or refractory cancer relies almost solely on administration of the treatment and observation of the clinical outcome. The research described in this proposal directly addresses each of these issues relevant to ovarian cancer. Considering that combinational therapy which includes cisplatin is a first-line treatment for ovarian cancer (5), our work will focus on cisplatin resistant ovarian cancers.

Body

Our original statement of work presented two specific aims of which one was to be accomplished in the first year of the grant period. We will report on our progress to date on the first specific aim and highlight the results of the experiments from each of the proposed tasks in the approved statement of work. The first specific aim was to "develop the methodologies to allow the analysis of proteins with an affinity for cisplatin-damaged DNA to be identified via proteomics technology." There were 2 tasks proposed each with 4 sub tasks. The first task involved development of affinity matrices for retention of proteins with an affinity for cisplatin-damaged DNA. We successfully developed a Sepharose based matrix and optimized the substitution of the cisplatin-damaged DNA by modifying the methods for preparation of the DNA. With greater degree of substitution of the DNA on the matrix, selectivity was increased and we can retain a large number of proteins specifically on the matrix and elute them in a small volume under relatively mild conditions. The results presented in figure 1 are a representative single dimension SDS PAGE analysis of cell free extracts prepared from ovarian cancer cells fractionated on the Cisplatin-damaged DNA Sepharose matrix. An aliquot of proteins loaded on the column is shown in lane 1 with the proteins that were not specifically bound eluted in fractions 2-4. The proteins specifically retained on the matrix were eluted with 0.4 M NaCl and are presented in lanes 5-7 and reveal a series of proteins ranging from 20-200 kDa. The first elution, lane 5 contains 90% of the eluted protein and was selected for further analysis. The efficiency of elution and the concentration of the eluted protein obtained are more that adequate for mass spectrometry analysis and we did not pursue the microlatex bead based affinity matrix.

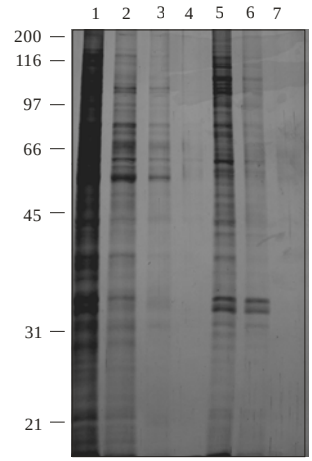


Figure 1. SDS Page analysis of protein fractions from a mini-cisplatin DNA Sepharose column. Aliquots from the cell extract (lane 2), unbound protein (lane 2-4) and specifically bound proteins (lane 5-7) were separated by SDS-PAGE and stained with silver.

The second subtask of Task 1 was to optimize the SELDI-TOF MS analysis of the fractionated ovarian cancer cell extract. The first elution fraction was used and protein spotted directly on a gold chip for analysis. Different energy absorbing matrices were tested and the results revealed that SPA consistently out performed CHCA and therefore is used unless noted otherwise. Samples of the eluted proteins were also processed for both solution and on chip trypsin digestion as detailed in the statement of work. The results presented in Figure 2 demonstrate that SELDI-TOF MS analysis of the undigested protein (Panel A) reveals a series of proteins with M/Z values ranging from 5 kDa to 20 kDa, effectively increasing the range protein sizes that can be analyzed. The intensity of the protein peaks, while clearly above background, was not near the maximum that can be detected. Therefore, to increase sensitivity and detection of proteins that "do not fly as well" in the SELDI-TOF MS trypsin digestion of the eluted proteins was performed. An in solution digestions was performed and the results are presented in Figure 2 panel B. As evident from the intensity and complexity of the spectrum, we have increased the sensitivity and a significant increase in peaks was observed, as expected. These results will allow us to have more determinants in the sensitive and resistant ovarian cancer fingerprint and may also allow the identification of specific proteins present in the protein pool eluted from the affinity matrix.

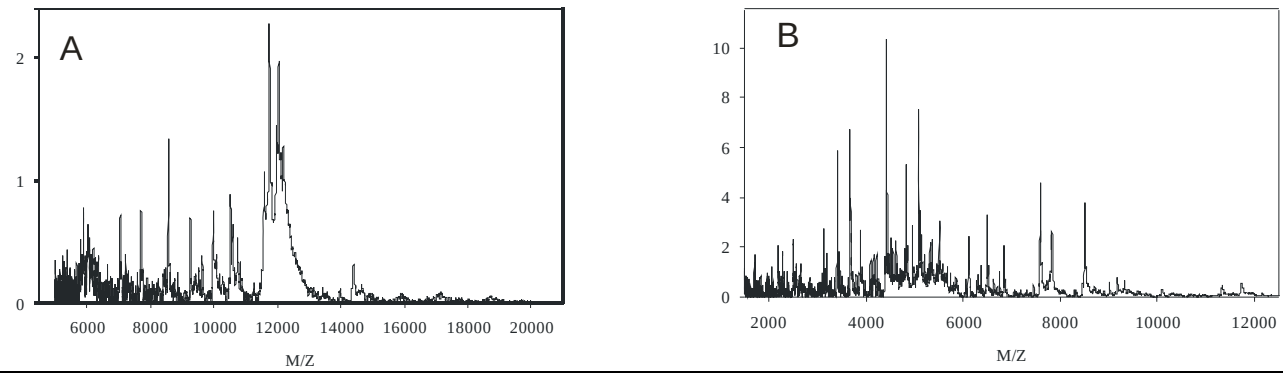


Figure 2. SELDI-TOF MS analysis of eluted protein. Protein from fraction 5 were analyzed directly (Panel A) or following exhaustive trypsin digestion (Panel B). The proteins were spotted onto a GOLD proteinCHIP and the EAM used was SPA.

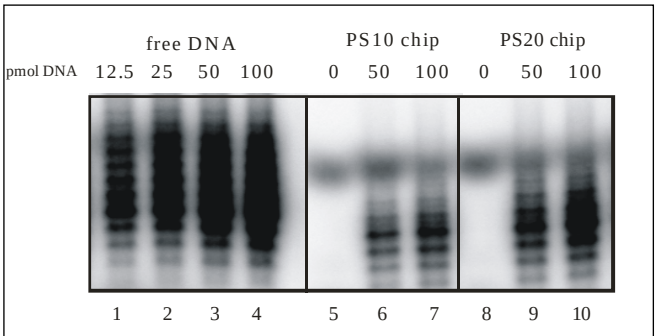


Figure 3. Terminal transferase labeling of DNA to determine binding efficiency. The concentrations of DNA indicated in the figure were either directly labeled with [a-P32]dGTP and terminal transferase (lanes 1-4) or following binding to the Ps10 (lanes 5-7) or Ps20 (lanes8-10 proteinCHIP. The signal in lanes 5-10 represent the DNA not bound to the CHIP following derivitization with the indicated amount of DNA.

The second main task to b accomplished in the first year of the grant was to determine the efficiency and utility of direct derivatization of activated SELDI-TOF chips with cisplatin modified DNA to analyze the proteins with an affinity for this DNA structure. We initially prepared DNA substrates with a 5' aminolinker modification. This modified DNA terminus with react with the activated matrix on the SELDI PS10 or PS20 CHIPS which both contain amino reaction chemistries. We compared the efficiency of each of these chemistries for reacting with the 5' aminolinker modified DNA. The results presented in Figure 3 shown the outcome of these analyses. The amount of DNA bound to the chip was determined by reacting a specific amount of DNA quantifying the amount of DNA that did not bind the spot. We employed terminal transferase labeling of the DNA with a radioactive label and separation of the products by DNA sequencing gel electrophoresis. Positive controls labeling know amounts of the substrate are presented in lanes 1-4 and show a clear titration with increasing DNA. The results in the DNA not bound by the PS10 and 20 chips indicate that a significant amount of the DNA was bound by the chips with the PS10 being more efficient than the PS20. The single stranded 30 base DNA substrates used in these analyses indicate a high degree of derivatization of the CHIP and the PS10 was used to analyze protein binding. Considering the nature of the DNA on the chip, a short single stranded DNA, we sought to validate the methodology with a protein known to have a high affinity for the DNA. Replication protein A (RPA) was purified and bound to the derivatized spots. The results obtained demonstrated that RPA binding to the chips was largely independent of derivatization of the spot with DNA. We are able to detect the 14 kDa subunit on spots with and without DNA. When extensive washing of the spots was performed to reduce the DNA independent binding of RPA to the CHIP we also lost binding to the spot derivatized with the DNA. These results indicate that RPA is not binding efficiently to the DNA on the chip. There are two possibilities we considered, first is that the DNA is too short to support binding and the second that the DNA may be bound to the chip by the nitrogenous bases in the DNA and therefore may restrict access to proteins. To overcome these issues we have employed a 5' biotin labeled DNA primer that was used in PCR reactions to amplify a 200 bp duplex DNA that contains a single 5' biotin. This DNA was then bound to a PS10 chip to which we derivatized streptavidin. This procedure effectively establishes a single attachment site for the DNA and a considerable longer DNA length to promote binding of the protein. Considering the nature of this DNA substrate we validated this CHIP methodology using purified Ku protein, which has a high affinity for duplex DNA ends. SELDI-TOF MS analysis is presented in Figure 4 and the results demonstrate that Ku is selectively retained on these spots. We also performed on chip trypsin digestion of the bound Ku and were able to identify numerous peptides of the Ku dimer, which increased the sensitivity of the detection.

During years 2 and 3 of funding we have made considerable progress towards completion of the aims of our proposal. I have broken down our progress by specific aim. Towards completion of aim 1, we have continued to refine and optimize the methodologies to allow proteomic analysis of DNA repair proteins via SELDI-TOF MS analysis.

Our procedure involves derivatizing a chemically modified MS target with a modified DNA and performing fractionation and analysis directly on the SELDI target. In our annual report for year 1, we presented data on the efficiency of derivatization of the SELDI CHIPS. Having DNA bound to the chip we the asked if the DNA able to be bound by cellular proteins. We therefore applied known DNA repair proteins, RPA (Figure 4) and Ku (Figure 5), to the DNA modified chips. Unbound protein was removed by three washed and the bound protein directly analyzed after the addition of EAM. The results shown in Figure 4 demonstrate the ability to retain these proteins of the derivatized proteinCHIP arrays. Importantly, BSA was included with the RPA and Ku and the inability to detect BSA demonstrates the specificity. Control experiments demonstrated that un-derivatized chips gave significantly reduced signal and heat denaturation of RPA abrogated binding.

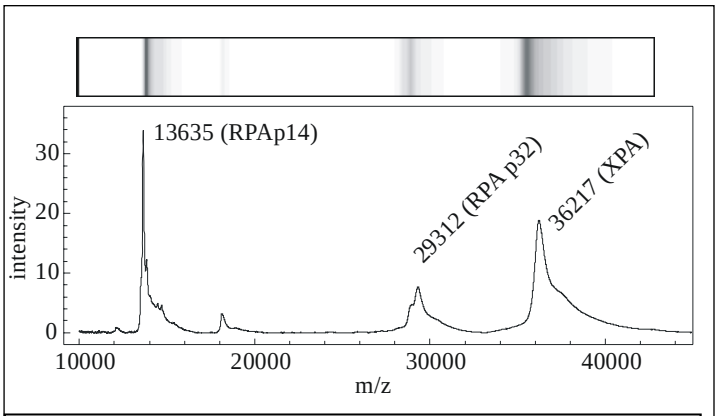


Figure 4. Retention of RPA on a DNA derivatized ProteinChip array. Purified RPA was applied to the ProteinChip and allowed to bind for 60 min at 25°C. The unbound protein was washed from the spot and allowed to dry. EAM was applied and the chip analysed in the PBSII SELDI-TOF MS. The top panel is an SDS gel representation of the spectrum obtained on the bottom panel.

The detection of these large proteins even in purified samples was very good though calculation revealed that a relatively small percentage of the applied protein was in fact retained by the modified DNA matrix. We also were able to observe the p70 subunit of RPA though the detection efficiency was considerable reduced compared to the p14, p34 and XPA proteins.

The analysis of the Ku protein binding to the DNA modified chip was event less efficient compared to RPA and XPA. The results presented in Figure 5 show a representative analysis. In this case while clearly detectable, the amount of Ku retained and detected was estimated to be 1% of the applied protein. The Ku 80 protein actually has a calculated mass of 86, consistent with the SELDI-TOF analysis. This relatively low level of signal is likely the results of the inefficiency of the desorption and ionization of the large proteins molecules.

Therefore we pursued on chip tryptic digestion of the retained proteins as described on our initial application. This proved to increase the sensitivity of the detection of each protein in addition to allowing the fingerprint of each protein to be determined. A representative analysis is presented in figure 6. In these experiments the proteins were bound to the DNA derivatized chip and unbound proteins removed by washing. The bound protein was then treated with trypsin at various concentrations on the chip and digestion performed for a range of time. The digestion reactions were terminated by the addition of EAM. The chips were placed in a humidified chamber to reduce evaporation of the small volumes used in these analyses. The increased sensitivity observed in the RPA Panel A, XPA panel B and the combination of the two proteins on a single spot Panel C

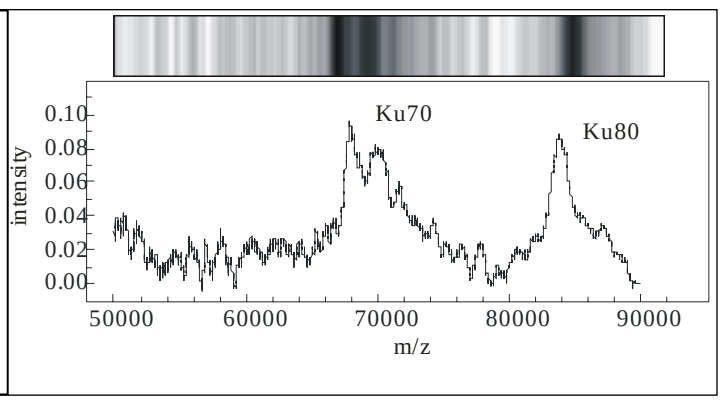


Figure 5. Retention of Ku on a DNA derivatized ProteinChip array. Purified Ku was applied to the ProteinChip and allowed to bind for 60 min at 25°C. The unbound protein was washed from the spot and allowed to dry. EAM was applied and the chip analysed in the PBSII SELDI-TOF MS. The top panel is an SDS gel representation of the spectrum obtained on the bottom panel.

is evident by the y-axis intensity values. Comparison of the spectra with that of purified RPA or XPA treated with trypsin in solution and them applied to the chip revealed a coincidence of peaks present in each spectra.

The next step with the application of cell extracts to the DNA derivatized chips. This step proved more difficult in that the sensitivity of the intact, undigested proteins was limited, as we demonstrated in the year one progress report where we were able to detect specific undigested proteins in the low molecular weight range (<15kDa). We pursued the analysis of these extracts and retained proteins on the chip by tryptic digestion, which again increased sensitivity, but also dramatically increased the complexity of the analysis. While peak identification analysis and computer searches where pursued we also undertook the definitive identification of the proteins retained on the DNA modified chip. We fractionated the extracts on DNA modified beads similar to the DNA modified SELDI CHIP and analyzed the retained proteins. The first methodology involved direct tryptic digestions of the protein pool and MALDI-TOF analysis. SELDI on the DNA modified CHIP was not used as the proteins were digested with trypsin and therefore have lost biologic activity. A representative analysis is presented in figure 7. The full MALDI-TOF spectrum is presented in the center panel and expanded in the peripheral sections. Clearly excellent signal intensity and resolution was observed.

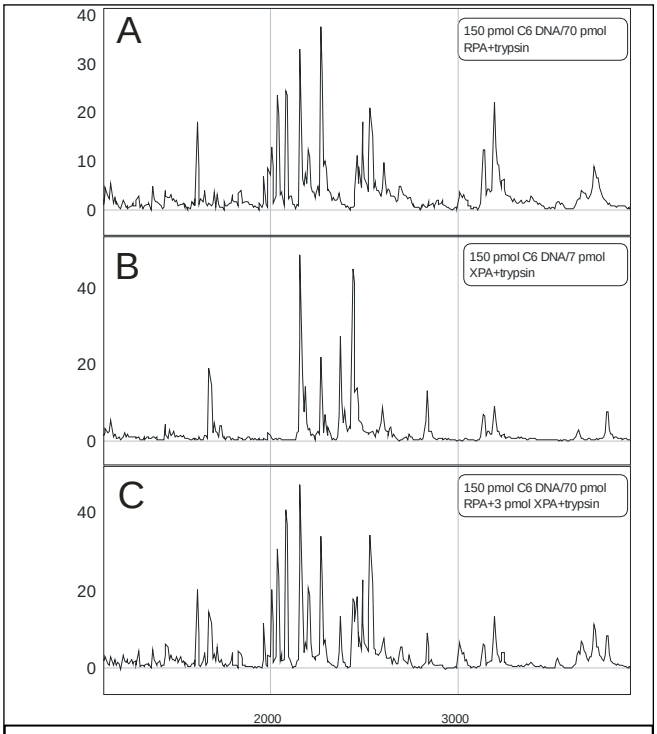


Figure 6. On-chip tryptic digestion of proteins retained on a DNA modified CHIP.

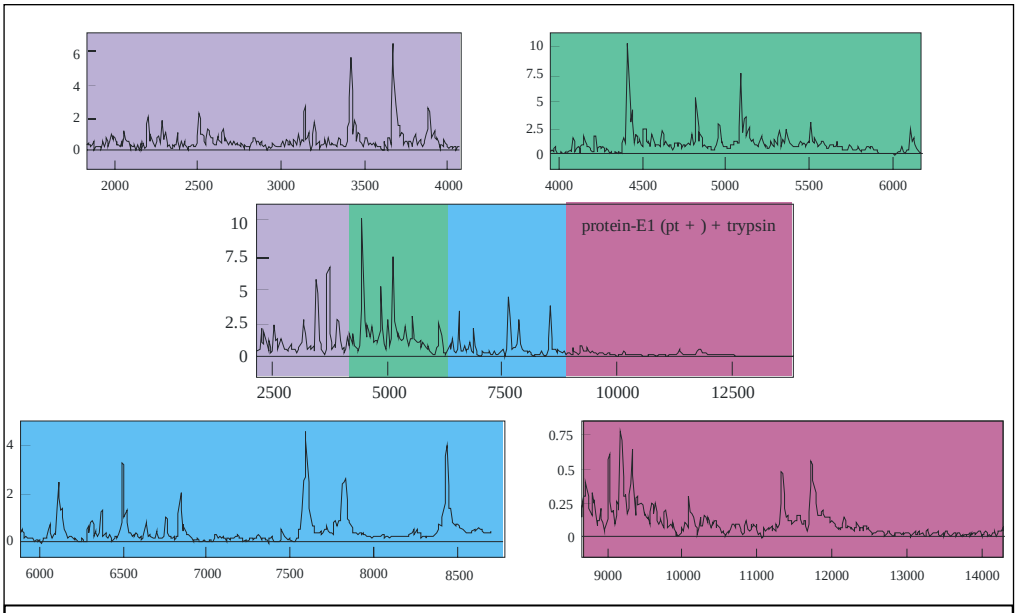
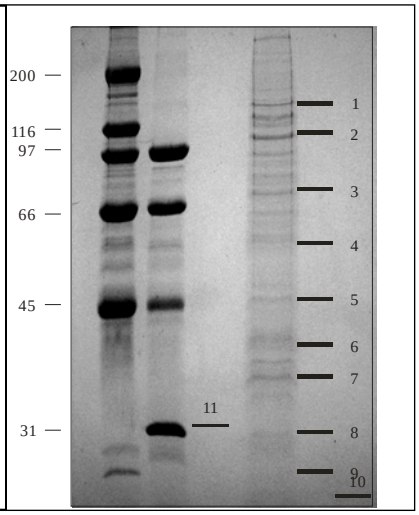


Figure 7. MALDI-TOF MS analysis of tryptic fragments from E1 fraction. MALDI-TOF MS analysis was performed as described earlier. Protein from the E1 fraction was digested with trypsin by incubation overnight at 37 C and peptide fragments analyzed. The center panel represents the entire spectrum collected and the outlying panels are expanded views of the indicated regions. The x-axis is M/Z and y-axis is the signal intensity.

Based on these results we then set out to identify the individual proteins. This was accomplished by SDS-PAGE separation and in-gel tryptic digestion of individual proteins. A representative example of an SDS gel is presented in Figure 8. Following digestions the peptides were analyzed by MALDI-TOF MS. A representative spectra is presented in Figure 9. The top panel represents the trypsin control and the middle panel digestion of the band from position 1 on the gel in Figure 8.

Each protein was processed independently and MASCOT searched performed to identify the individual proteins. The list to date is presented in Tables 1 and 2.

Figure 8. Identification of individual cisplatin-DNA binding proteins. SDS-PAGE analysis of fraction E1 detected by colloidal blue staining. The bands corresponding to each numbered line were excised. Controls bands 10 and 11 represent a blank lane and the 32kDa carbonic anhydrase protein, respectively. The gel slices were processed for in-gel trypsin digestions as described earlier.



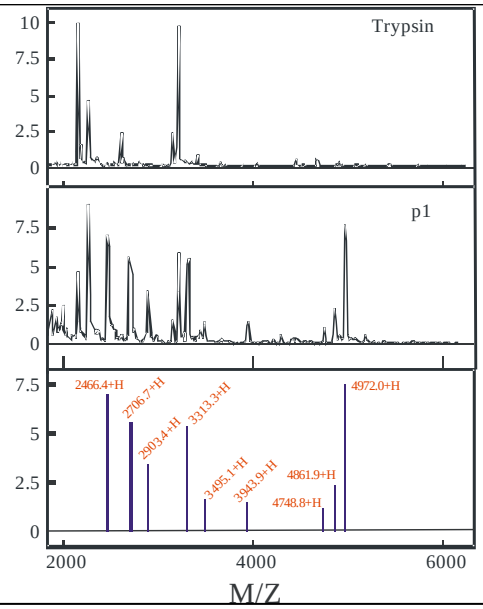


Figure 9. The top panel is the MS analysis of band 10 and represents a trypsin control. The middle panel in the spectrum from band 1 and the bottom panel represents the analysis of the peaks specific to band 1 subtracting those present in both spectrum.

Band	# peaks	min. match	tolerance (Da)	mw range (kDa)
1	9	6	4	135-165
1	20	11	4	135-165
3	19	9	4.5	66-80
4	11	5	4.5	49-61
8	19	7	4.5	26-32
11	23	9	2.5/4.5	24-36

Table 1.

BAND	# matches	ID	Description
1	12	DPG1_HUMAN	DNA polymerase gamma
	12	MSH6_HUMAN	MutS alpha 160 kDa subunit
	12	FACA_HUMNA	Fancon anemia group A protein
3	4	FXR2_HUMAN	Fragile X syndrome related protein 2
4	4	MDM4_HUMAN	MDM4 p53 binidng protein
8	6	RA51_HUMAN	Rad51 splice isoform 2
	7	FKB7_HUMAN	FK506 binding protein 7
11	9	CAH2_Bovine	Bovine carbonic anhydrase

Table 2.

In addition we have employed a modification of the procedures that allows higher throughput and employs biotin modified DNA and a strepavidin matrix. The proteins identified in this manner have confirmed our original analyses and is being pursued for the other cell lines. These analyses are being continued to validate the initial spectra and will be completed in the coming year using existing funds in the no-cost extension.

Key research Accomplishments

- Established column fractionation procedures for retention and elution of cisplatin-damaged DNA binding proteins.
- Established procedures for SELDI-TOF MS analysis of the eluted proteins including “on chip” and in solution trypsin digestion of the eluted proteins.
- Initiated MS analysis of the cisplatin-damaged DNA binding proteins in the A2780 series of ovarian cancer cells.
- Established methodologies for “on chip” selective retention of DNA binding proteins using streptavidin derivatization of the protein chip and the oriented binding of biotin labeled DNA.
- Established “on chip” trypsin digestion of proteins retained on the DNA modified chip.
- Established protocol for biotin-streptavidin isolation of DNA damage recognition proteins and their analysis of MALDI-TOF MS.

- Identified the mcm2 and RAD51 protein in the selected pool of DNA damage proteins and confirmed selection by western blot analysis.

Reportable outcomes

1. Jason A. Lehman and J**ohn J. Turchi.** MALDI-TOF Mass Spectrometry Analysis of the Human Ku Heterodimeric Protein for DNA-Binding Regions**.** 6th Annual Midwest DNA repair, Lexington KY, June 2003
2. Jiazhen Wang and **John J. Turchi.** Analysis of DNA Repair Protein Expression in Cisplatin-Resistant Ovarian Cancers by SELDI-TOF Mass Spectrometry. 6th Annual Midwest DNA repair, Lexington KY, June 2003
3. Brooke Andrews, Jason Lehman and John Turchi. (2006) Kinetic analysis of the Ku-DNA binding activity reveals a redox-dependent alteration in protein structure that stimulates dissociation of the Ku-DNA complex. Journal of Biological Chemistry, 281, 13596-13603.
4. **John J. Turchi** (2006) Nitric oxide and cisplatin resistance: NO easy answers. Proceedings of the National Academy of Sciences, 103, 4337-4338.
5. **Jiazhen Wang.** Analysis of protein expression in cisplatin-resistant ovarian cancers by MALDI-TOF mass spectrometry. M.S. Thesis, Wright State University. 2005
6. Grant Application to the Flight Attendants Medical Research Institute**.** Analysis of DNA repair capacity to predict and target chemoresistant small cell lung cancer. PI John J Turchi. Funded 6/06-5/09. This application proposes to continue to develop the methodology initiated in the OCRP award to identify DNA repair proteins and correlate their expression and activity with clinical resistance to cisplatin in a lung cancer model.

Personnel receiving support: Jason Lehman, Ph.D Candidate, Jiazhen Wang M.S.

Conclusions

The research completed under this award has allowed us to determine numerous critical parameters associated with detection of DNA repair proteins in tissues via mass spectrometry. The approach of affinity separation coupled with MS detection has over the course of this research been greratly aided by advanced in MS technology. The Ciphergen SELDI MS used today is more sensitive and accurate allowing a greater degree of certainty in the analysis of individual proteins. Likewise more recent advances in on chip methodologies developed by us under this grant award has increased the sensitivity of detecting the individual proteins. We are therefore continuing to pursue this technology to obtain further proof-of-principle that DNA repair protein expression and activity are indicative of cisplatin sensitivity in cancer. The difficulties in obtaining consistent analyses with tissue culture cells will only be exacerbated when analysis of human tumor samples is pursued. Therefore, we are also expanding the technology to use a 96-well ELISA format. Uniformity of the preparation and handling of the tissue, separation and processing of the proteins all we be critical in these analysis. Thus standardaized procedures based on the methodologies obtained under this award will continue to be developed. Finally, we have also identified known proteins with potentially novel roles in cisplatin resistance using affinity separation coupled with MS detection. These novel roles will be pursued to determine if in fact they contribute to resistance and have any predictive value.

Reference List

1. Perez, R. P., Godwin, A. K., Hamilton, T. C., and Ozols, R. F. (1991) *Semin. Oncol.* **18,** 186-204
2. Mills, G. B., Bast, R. C. J., and Srivastava, S. (2001) *J. Natl. Cancer Inst.* **93,** 1437-1439
3. Srinivas, P. R., Srivastava, S., Hanash, S., and Wright, G. L. J. (2001) *Clin. Chem.* **47,** 1901-1911
4. Boente, M. P., Hamilton, T. C., Godwin, A. K., Buetow, K., Kohler, M. F., Hogan, W. M., Berchuck, A., and Young, R. C. (1996) *Curr. Probl. Cancer* **20,** 83-137
5. McGuire, W. P. and Ozols, R. F. (1998) *Semin. Oncol.* **25,** 340-348